DECLARATION

I, Masanori Hirota of HIROTA AND ASSOCIATES, residing at Wakabayashi Bldg. 3F, 8-5, Akasaka 2-chome, Minato-ku, Tokyo 107-0052, Japan, do hereby certify that I am conversant with the English and Japanese languages and am a competent translator thereof, and I further certify that to the best of my knowledge and belief the following is a true and correct translation made by me of the document in the Japanese language filed for a patent application in Japan under No. 11/309,238 on October 29, 1999 in the name of JAPAN SCIENCE AND TECHNOLOGY CORPORATION at Tokyo, Japan, entitled: "BACTERIAL CELL COMPONENT-UNRESPONSIVE MODEL MOUSE".

Signed this 12 day of May, 2003

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[Title of the Invention] BACTERIAL CELL COMPONENT-UNRESPONSIVE MODEL MOUSE

[Scope of Claims]

[Claim 1] A non-human animal characterized by that its function of TLR2 gene is deficient on its chromosome.

[Claim 2] The non-human animal according to claim 1, wherein the non-human animal is a rodent.

[Claim 3] The non-human animal according to claim 2, wherein the rodent is a mouse.

[Claim 4] A TLR2 knockout mouse characterized in being obtainable by a process comprising the steps of: a targeting vector is constructed by replacing a whole or a part of a gene fragment of an exon region containing a cytoplasmic region of TLR2 gene obtained by screening a mouse genomic library with a probe derived from a mouse EST clone with a plasmid having a poly A signal and a marker gene; the targeting vector is linearized and then introduced into an embryonic stem cell; chimeric mice are generated by microinjecting the targeting ES cells whose function of TLR2 gene is deficient into the blastocysts of mice; heterozygous mice are generated by mating the chimeric mice and wild-type mice; the heterozygous mice are interclossed.

[Claim 5] A non-human animal characterized in being unresponsive to peptidoglycan.

[Claim 6] A non-human animal characterized in being hyporesponsive to a cell wall fraction of Gram-positive bacteria.

[Claim 7] The non-human animal according to claim 5 or 6, wherein the non-human animal is a rodent.

[Claim 8] The non-human animal according to claim 7, wherein the rod nt is a mouse.

[Claim 9] A screening method of an agonist or an antagonist of TLR2 characterized in comprising the steps of: a subject material is administered to a TLR2 knockout mouse and a wild-type mouse; the activity level of the macrophages or of the splenocytes derived from the TLR2 knockout mouse and from the wild-type mouse are compared and assessed.

[Claim 10] A screening method of an agonist or an antagonist of TLR2 and/or TLR4 characterized in comprising the steps of: a subject material is administered to a TLR2 knockout mouse and a TLR4 knockout mouse; the activity level of the macrophages or of the splenocytes derived from the TLR2 knockout mouse and from the the TLR4 knockout mouse are compared and assessed.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to a non-human animal whose function of TLR2 gene is deficient on its chromosome, a non-human animal being unresponsive to peptidoglycan and being deficient in function of TLR2 gene, a non-human animal characterized in being hyporesponsive to a cell wall fraction of Gram-positive bacteria, and a screening method of an agonist or an antagonist of TLR2 and the like with these materials.

[0002]

[Prior Art]

It has been known that a Toll gene is required to control dorsoventral patterning during the embryonic development of Drosophila (Cell 52, 269-279,1988, Annu. Rev. C ll Dev. Biol. 12, 393-416, 1996), and for antifungal immune responses in adult

fly (C ll 86, 973-983, 1996). It has been clarifi d that the Toll is a type I transmembrane receptor with an extracellular domain containing leucine-rich repeat (LRR) and that its cytoplasmic domain shows high homology to that of mammalian interleukin-1 recepter (IL-1R)(Nature 351, 355-356, 1991, Annu. Rev. Cell Dev. Biol. 12, 393-416, 1996, J. Leukoc. Biol. 63, 650-657, 1998). It has been also clarified that another Toll family member, 18-wheeler, participates in the antibacterial host defense but not in the antifungal immune response, and that particular pathogens induce specific antimicrobial immune responses in Drosophila through the selective activation of the Toll pathways (Proc. Natl. Acad. Sci. USA 94, 14614-14619, 1997, EMBO J. 16, 6120-6130, 1997, Curr. Opin. Immunol. 11, 13-18, 1999).

[0003]

Recently, mammalian homologs of Toll, designated as Toll-like receptors (TLRs), have been identified, and so far, six families including TLR2 and TLR4 have been reported (Nature 388, 394-397, 1997, Proc. Natl. Acad. Sci. USA 95, 588-593, 1998, Blood 91, 4020-4027, 1998, Gene 231, 59-65, 1999). It has been known that the TLR families, as in the case of the IL-1R, recruit IL-1R-associated kinase (IRAK) through an adaptor protein MyD88 and activate TRAF 6, and then activate NF-$_{\kappa}$B in the downstream (J. Exp. Med. 187, 2097-2101, 1998, Mol. Cell 2, 253-258, 1998, Immunity 11, 115-122, 1999). Further, the role of the TLR families in mammals is also believed to participate in innate immune recognition as pattern recognition receptors (PRRs), which recognize bacterial cell common structures (Cell 91, 295-298, 1997).

[0004]

It has been reported that one of such pathogen-associated mol cular patterns (PAMPs) to be recognized by the PRRs is lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria (Cell 91, 295-298, 1997), that said LPS stimulates host cells and makes them produce various proinflammatory cytokines including TNFα, IL-1, and IL-6 (Adv. Immunol. 28, 293-450, 1979, Annu. Rev. Immunol. 13, 437-457, 1995), and that the LPS captured by LPS-binding protein (LBP) is delivered to CD14 on the cell surface (Science 249, 1431-1433, 1990, Annu. Rev. Immunol. 13, 437-457, 1995). However, since CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein without a transmembrane domain, the existence of a bona fide signaling receptor of LPS has been believed.

【0005】

TLR4, which belongs to the TLR family, is a signaling molecule of LPS, which is a bacterial cell component of Gram-negative bacteria, and transfection of the TLR4 leads to a low constitutive activation of NF-$_\kappa$B (J. Exp. Med. 188, 2091-2097, 1998, Nature 395, 284-288, 1998). On the other hand, as TLR2 transmits LPS signal when overexpressed in human embryonic kidney 293 cells in vitro, TLR2 has been thought to be a candidate for the LPS receptor. In addition, Godawski's group has reported that human TLR2 could interact with CD14 to form the LPS receptor complex (J. Immunol. 163, 639-643, 1999). Stimulation treatment with LPS leads to oligomerization of receptors and to subsequent recruitment of IRAK to the receptor complex. In contrast, groups of Poltorak and Qureshi have report d that TLR4 is the causative gene of the LPS hyporesponsiven ss of C3H/HeJ mic , that is, the Lps gen ,

according to positional cloning (Science 282, 2085-2088, 1998, J. Exp. Med. 189, 615-625, 1999).

[0006]

The inventors of the present invention have found by generation of TLR4-deficient mice that TLR4 is actually involved in LPS signaling (J. Immunol. 162, 3749-3752, 1999). The findings may be attributed to species-specific differences in the primary structure of TLR, in other words, LPS signaling could be mediated by TLR4 in mice and by TLR2 in humans. However, there is a report showing that mouse TLR2 also activated NF-$_{\kappa}$B in response to LPS (J. Immunol. 162, 6971-6975, 1999). In addition, Chow et al. have reported that they obtained the result showing that human TLR4 activated NF-$_{\kappa}$B-mediated gene expression by stimulation to LPS/CD14 in a dose-dependent or a time-dependent manner, which is consistent with the observation of C3H/HeJ mice, whereas they obtained the result conflicting with that of Kirschning's group when human 293 cells were used, and they have speculated that the differences of outcome may be due to differences in the lot of 293 cells as well (J. Biol. Chem. 274, 10689-10692, 1999).

[0007]

Recently, it has been reported that TLR2 may not be involved exclusively in responsiveness to LPS derived from Gram-negative bacteria (J. Immunol. 162, 6971-6975, 1999) but may also act as a signaling receptor for peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria , which have another common bacterial structural pattern (J. Biol. Chem. 274, 17406-17409, 1999, J. Immunol. 163, 1-5, 1999). Further, it has b n also report d that whole Gram-positive bacteria, soluble PGN, and LTA induced the activation of NF-$_{\kappa}$B in 293 cells

expressing TLR2, but not induced the activation of NF-$_{\kappa}$B in the cells expressing TLR1 or TLR4 (J. Biol. Chem. 274, 17406-17409, 1999). Still further, it has been also reported that Chinese hamster ovary (CHO) fibroblast cells which express human TLR2 but not TLR4 were activated similarly by heat-killed Staphylococcus aureus and Streptococcus pneumoniae, and PGN derived from Staphylococcus aureus (J. Immunol. 163, 1-5, 1999).

[0008]

On the other hand, lipoproteins/lipopeptides from mycobacterium and Borrelia burgdorferi induced the activation of host cells through TLR2 in vitro (Science 285, 736-739, 1999, Science 285, 732-736, 1999). Nevertheless, the conclusions obtained from overexpression experiments do not necessarily reflect the function of TLR family in vivo. It is also reported that the results of analysis of the responsiveness based on NF-$_{\kappa}$B activation are not related to biological responses mediated by these stimuli (Infect. Immun. 66, 1638-1647, 1998).

[0009]

[An Object to be Attained]

Though in vivo responses to bacterial cell components are expected to vary depending on the difference of expression levels of each TLR on the cell surface, the contribution of individual members of the TLR family to signaling by bacterial cell components' stimuli in vivo remains to be elucidated. An object of the present invention is to provide a non-human animal whose function of TLR2 gene is deficient on its chromosome, a non-human animal being unresponsive to peptidoglycan and being deficient in function of TLR2 gene, a non-human animal characterized in being hyporesponsive to a cell wall fraction

of Gram-positive bacteria, and a screening method of an agonist or an antagonist of TLR2 and the like with these materials, which are useful for elucidating the contribution of individual members of the TLR family to signaling by bacterial cell components' stimuli in vivo, in particular, the role of TLR2 in vivo, and to provide a screening method and the like of a suppressor or a promoter of bacterial infection and an agonist or an antagonist for TLR2 with said bacterial cell component-unresponsive model non-human animals.

[0010]

[Means to Attain the Object]

The inventors of the present invention have conducted intensive study for attaining the object. They generated TLR2 gene-deficient mice as follows: an exon region including a cytoplasmic region of TLR2 gene is replaced with the neomycin-resistant gene by homologous recombination with plasmid vectors in ES cells and HSV-tk gene was induced into C-terminal side, and ES cell clones doubly resistant of G418 and gancyclovir were screened; the ES cell clones were microinjected into blastocysts of C57BL/6 mice; TLR2 knockout mice whose function of TLR2 genes is deficient were born through the germline at the expected Mendelian ratios. Then the inventors have found that those TLR2 knockout mice are transgenic mice which grow healthy and show no obvious abnormalities until 20 weeks of age, and that those TLR2 knockout mice are unresponsive to peptidoglycan, which is a cell wall component of Gram-positive bacteria, and the present invention has thus completed.

[0011]

The present invention relates to a non-human animal

characteriz d by that its function of TLR2 gene is deficient on its chromosome (claim 1), the non-human animal according to claim 1, wherein the non-human animal is a rodent (claim 2) and the non-human animal according to claim 2, wherein the rodent is a mouse (claim 3).

[0012]

The present invention also relates to a TLR2 knockout mouse characterized in being obtainable by a process comprising the steps of: a targeting vector is constructed by replacing a whole or a part of a gene fragment of an exon region containing a cytoplasmic region of TLR2 gene obtained by screening a mouse genomic library with a probe derived from a mouse EST clone with a plasmid having a poly A signal and a marker gene; the targeting vector is linearized and then introduced into an embryonic stem cell; chimeric mice are generated by microinjecting the targeting ES cells whose function of TLR2 gene is deficient into the blastocysts of mice; heterozygous mice are generated by mating the chimeric mice and wild-type mice; the heterozygous mice are interclossed (claim 4).

[0013]

The present invention further relates to a non-human animal characterized in being unresponsive to peptidoglycan (claim 5), a non-human animal characterized in being hyporesponsive to a cell wall fraction of Gram-positive bacteria (claim 6), the non-human animal according to claim 5 or 6, wherein the non-human animal is a rodent (claim 7) and the non-human animal according to claim 7, wherein the rodent is a mouse (claim 8).

[0014]

The present invention still further relates to a

screening method of an agonist or an antagonist of TLR2 characteriz d in comprising the steps of: a subject material is administered to a TLR2 knockout mouse and a wild-type mouse; the activity level of the macrophages or of the splenocytes derived from the TLR2 knockout mouse and from the wild-type mouse are compared and assessed (claim 9) and a screening method of an agonist or an antagonist of TLR2 and/or TLR4 characterized in comprising the steps of: a subject material is administered to a TLR2 knockout mouse and a TLR4 knockout mouse; the activity level of the macrophages or of the splenocytes derived from the TLR2 knockout mouse and from the the TLR4 knockout mouse are compared and assessed (claim 10).

[0015]

[Mode for Carrying out the Invention]

In the present invention, "a non-human animal whose function of TLR2 gene is deficient on its chromosome" means that a non-human animal wherein a part of or a whole of TLR2 gene on a chromosome is inactivated by disruption, deficiency, substitution or the like, and the function to express TLR2, which is expressed in wild-types, is lost. Specific examples of a non-human animal whose function of TLR2 gene is deficient on its chromosome include a rodent such as a mouse, a rat or the like, but it is not limited to these examples.

[0016]

The term "a wild-type non-human animal" in the present invention means a non-human animal being the littermate of the non-human animal whose function of TLR2 gene is deficient on its chromosome. For example, in case of mice, it means TLR2-nondeficient type litt rmate mice among F2 mice generated at the expected Mendelian ratio. When the deficient type and

the wild-type littermates of these F2 mice are used for experiments simultaneously, it becomes possible to conduct precise comparative experiments at individual level. With an example of knockout mice which have deficiency in TLR2, a generating method of the non-human animal whose function of TLR2 gene is deficient on its chromosome will now be explained.

[0017]

TLR2 gene can be cloned by amplifying a mouse genomic library by PCR or other methods with a probe derived from a mouse EST clone or the like. By DNA recombination technique, a part of or a whole of this cloned TLR2 gene, for example, a part or a whole of an exon region containing a cytoplasmic region of TLR2 gene are replaced with a poly A signal and a marker gene such as a neomycin resistance gene or the like, a targeting vector is constructed by inducing genes such as diphtheria toxin A fragment (DT-A) gene or herpes simplex virus thymidine kinase (HSV-tk) gene or the like into 5'-terminal side, this constructed targeting vector is linearized, and introduced into embryonic stem cells (ES cells) by electroporation method or the like, then cultured, and subsequently Es cells achieving homologous recombination by G418, ganciclovir (GANC) or other such antibiotics are selected. It is preferable to confirm whether these selected ES cells are the object recombinants by Southern blot analysis or the like.

[0018]

Chimeric mice can be obtained by microinjecting the recombined ES cells into blastocysts of mice, and put the blastocysts back into uteri of recipient mice. Under high chimeric ratio, there will be born much mor male chimeric mice than female ones. In such case, heterologous recombinant mice

(+/-: F1) are generated by interclossing the chimeric mice with female wild-type mice, and the homologous recombinant mice [F2; wild-type mice (+/+), TLR2 knockout mice (-/-)] can be obtained by mating the heterologous recombinant male mice and female mice. All of these mice are generated at the expected Mendelian ratio. As the method of confirming whether TLR2 knockout mice of the present invention are born, for example, the method wherein RNA is isolated from peritoneal macrophages of mice obtained by the above-stated method, and is examined by Northern blot analysis or the like, and the method wherein the expression of TLR2 in the mice is examined by Western blot analysis or the like are exemplified.

[0019]

It is possible to confirm that the obtained TLR2 knockout mice are unresponsive to peptidoglycan, which is a cell wall component of Gram-positive bacteria or the like, and are hyporesponsive to a cell wall fraction of Gram-positive bacteria, for example, by measuring the induction of IFN, proliferative response of splenocytes, the expression of MHC class II antigen on the surface of splenic B cells, in macrophages or splenic B cells of TLR2 knockout mice, in the presence of peptidoglycan, which is a cell wall component of Gram-positive bacteria or the like.

[0020]

Macrophages and splenocytes of TLR2 knockout mice of the present invention are unresponsive to peptidoglycan, and hyporesponsive to cell wall fractions of Gram-positive bacteria, and responsive to LPS, LTA and IL-4. Therefore, the TLR2 knockout mice can be used as useful model for elucidating action mechanisms of peptidoglycan, cell wall fractions of Gram-

positive bacteria or the like.

[0021]

The present invention makes it possible to conduct the screening of an agonist or an antagonist to TLR2 by administering a subject material to the TLR2 kcnokout mice and wild-type mice, for example, by administering the subject material directly to the mice, or by contacting the subjected material with intraperitoneal macrophages and splenocytes derived from the mice and by detecting, comparing and assessing the activity levels of macrophages or splenocytes. Further, the present invention makes it possible to conduct the screening of an agonist or an antagonist to TLR2 and/or TLR4 by administering a subject material to each of TLR2 and TLR4 knockout mice, and to wild-type mice if necessary, and by comparing and assessing the activity levels of macrophages or splenocytes derived from the TLR2 knockout mice and the TLR4 knockout mice.

[0022]

[Examples]

The present invention will be explained more specifically with examples below, but the technological scope of the present invention is not limited to these examples.

Reference (Generation of TLR4-deficient mice)

In order to compare responsiveness of TLR2- and TLR4-deficient mice to cell wall components of Gram-positive and Gram-negative bacteria, TLR4-deficient mice ($(F_2$ interbred from 129/OlaXC57BL/6) were generated by gene targeting as described previously (J. Immunol. 162, 3749-3752, 1999) by the inventors of the present invention.

[0023]

Example 6 (Generation of TLR2 knockout mice)

TLR2 gene was screened from 129/SvJ mouse genomic library (Stratagene) using a probe derived from a mouse EST clone (accession number D77677) similar to human TLR2 gene, and subcloned into pBluescript vector (Stratagene), then characterized by restriction enzyme mapping and DNA sequencing. A targeting vector was constructed by replacing a gene fragment at an exon region 1.3 kb containing cytoplasmic domain of TLR2 gene with pMC1-neo (Stratagene) having Poly A signal. The targeting vector was flanked by a 4.8 kb 5' genomic fragment and a 1.0 kb 3' fragment and contained an HSV-tk cassette at the 5' terminal. The targeting vector was linearized with SalI and electroporated into E14.1 embryonic stem cells (ES cells). 120 clones resistant to G418 and gancyclovir were screened for homologous recombination by PCR and 9 clones were confirmed by Southern blot analysis using the probe indicated in Figure 1A.

[0024]

Chimeric mice were generated by microinjection of 3 targeted ES clones containing a mutant TLR2 allele into blastocysts of C57BL/6 mice. Male chimeric mice were bred to C57BL/6 females to produce heterozygous mice. The heterozygous mice were intercrossed to obtain homozygotes (Fig 1B). TLR2-deficient mice of the present invention could be generated at the expected Mendelian ratio, and did not show any obvious abnormality until 20 weeks.

[0025]

To confirm that the mutation caused inactivation of the

TLR2 gene, total RNA (15 μg) was extracted from peritoneal macrophages (5×10^6) of wild-type (+/+) and TLR2 knockout (-/-) mice and then electrophoresed, transferred to a nylon membrane, and Northern blot analysis was conducted using cDNA specific to [^{32}P]-labelled TLR2, or cDNA specific to GAPDH (glycelaldehyde-3-phosphate dehydrogenase) as the method previously described (Immunity 9, 143-150, 1998). As a result, TLR2 mRNA was not detected in peritoneal macrophages of TLR2-deficient mice (Fig 1C). In addition, it was shown that the expressions of CD3, B220, CD4, and CD8 in thymocytes and splenocytes of TLR2 knockout mice were not different from those of wild-type mice (data not shown).

[0026]

Example 2 (Responsiveness of TLR2 knockout mice to endotoxin)

1 mg of LPS derived from Escherichia coli (O55:B5) was injected into each of TLR2 knockout mice (n=5), TLR4 knockout mice (n=5) and wild-type mice (n=5) of the present invention, and LPS unresponsiveness was examined by their survival rate. The results are shown in Fig. 2. Fig. 2 confirms that though TLR2 knockout mice (TLR2-/-) and wild-type mice of the present invention responded to LPS and almost all of them died within 4 days after injection, none of TLR4 knockout mice (TLR4-/-) died even after 6 days after injection, and that TLR4 knockout mice are unresponsive to endotoxin.

[0027]

Example 3 (Responsiveness of TLR2 knockout mice to cell components of Gram-negative bacteria)

Each of TLR2 knockout (TLR2-/-), TLR4 knockout (TLR4-/-) and wild-type (wild-type) mice w re intraperitoneally injected with 2 ml of 4% thioglycollate medium (DIFCO). Three

days later, peritoneal exudate cells were isolated from the periton al cavity of each mouse. These cells were cultured in RPMI1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) for 2 hours at 37° C and washed with ice-cold Hank's buffered salt solution (HBSS; GIBCO) to remove nonadherent cells. Adherent cells were used as peritoneal macrophages for following experiments.

[0028]

Each of obtained peritoneal macrophages were cultured for 24 hours with 1.0 ng/ml of synthetic lipid A derived from Escherichia coli (compound 506; Daiichi Pure Chemicals) or LPS derived from Salmonella minnesota Re-595 (Sigma) in the presence or absence of IFN-γ (30 unit/ml). Synthetic lipid A, which was soluble in endotoxin-free water and containing 0.025% of triethylamine, was used as said synthetic lipid A. After the culture, production amounts of IL-6 (Fig. 3A), TNFα (Fig. 3B) and NO_2^- (Fig. 3C) in culture supernatants were measured. Production amount of IL-6 was measured by enzyme-linked immunosorbent assay (ELISA; ENDOGEN), and that of TNFα was measured by ELSIA, according to manufacturer(Genzyme)'s instructions, and that of NO_2^- was measured by the Greiss method using NO_2/NO_3 Assay Kit (Dojindo Laboratories).

[0029]

These results indicate that macrophages of wild-type mice and TLR2 knockout mice showed similar responsiveness to LPS and lipid A, and produced IL-6 and TNFα, and it was confirmed that production of TNFα would be further increased when IFNγ was added to LPS or lipid A before the culture. By contrast, macrophages of TLR4 knockout mice produced neither IL-6 nor TNFα. Further, production of NO_2^- was confirmed by culturing

macrophag s of wild-type and TLR2 knockout mice with IFNγ-added lipid A or LPS. The obtained r sults were sam as those aforementioned even in the case the injection amount of lipid A or LPS was arranged to be 1 μg/ml (data not shown).

[0030]

Next, each of peritoneal macrophages of wild-type, TLR2 knockout and TLR4 knockout mice were cultured in the presence of LPS derived from Salmonella minnesota Re-595 at various concentrations shown in Fig. 17D, and production of TNFα was measured. The results indicate that macrophages of wild-type mice and TLR2 knockout mice showed similar tendency to increase in response to LPS in a dose-dependent manner, while macrophages of TLR4 knockout mice produced no TNFα in response to any concentration of LPS.

[0031]

Example 4 (Responsiveness to LPS of Salmonella minnesota Re-595)

Responsiveness of splenocytes of various mice (wild-type, TLR2-/- and TLR4-/-) to LPS of Salmonella minnesota Re-595 were examined. Splenocytes (1×10^5) of each mouse were isolated and then cultured and stimulated in 96-well plates with various concentrations of LPS shown in Fig. 4A. 1 μCi of [^{3}H]-thymidine (DuPont) was added 40 hours after the onset of the culture, and cells were further cultured for 8 hours, then [^{3}H] uptake was measured by a β scintillation counter (Packard) (Fig. 4A). As a result, the cell proliferative response was promoted in response to LPS in a dose-dependent manner in splenocytes of wild-type and TLR2 knockout mice as well. By contrast, whatever the concentration of LPS as a stimulus would be, no LPS-induced cell proliferativ response was obs rved in splenocytes of

TLR4-deficient mice.

[0032]

In addition, the expression of major histocompatibility complex (MHC) class II (I-A^b) on the surface of B cells in response to Re-595 LPS was examined by flow cytometry. Splenic B cells (1×10^5) of each of wild-type, TLR2 knockout (2-/-) and TLR4 knockout (4-/-) mice were isolated and cultured for 48 hours in 96-well plates with various concentrations (0, 10^1, 10^2, 10^3, 10^4 or 10^5 ng/ml) of LPS or 100 U/ml of IL-4. After the culture, the cells were collected and stained by combining I-A^b molecule on the surface of the cells and FITC-labelled antibody which is constructed by combining phycoerythrin (PE; PharMingen)-conjugated anti-B220 antibody or biotinylated anti-mouse I-A^b antibody (PharMingen) and fluorescein isocyanate (FITC; PharMingen)-conjugated streptavidin. The stained cells were analyzed on fluorescence-activated cell sorter Calibur (FACS Calibur) using CELLQuest software (Becton Dickinson) (Fig. 4B). As a result, Re-595 LPS enhanced the expression of I-A^b molecule on the surface of splenic B cells of wild-type and TLR2 knockout mice. In contrast, I-A^b molecule expression in splenic B cells of TLR4-deficient mice were not enhanced by Re-595 LPS, even when stimulated with high concentration of LPS (10^5 ng/ml). The above-mentioned results indicate that TLR2 knockout mice show responsiveness to LPS as wild-type mice did. When stimulated with IL-4, each knockout mice show normal expression of I-A^b molecule on the surface of splenic B cells.

[0033]

Example 5 (Unresponsiveness of macrophages of TLR2 knockout mic to cell wall components derived from Gram-positive

bacteria)

Responsiveness of each p riton al macrophages of said wild-type (wild-type), TLR2 knockout (TLR2-/-), TLR4 knockout (TLR4-/-) mice and the like to cell wall components derived from Gram-positive bacteria were examined with prepared cell wall specimens of S. aureus, C. diphtheriae and N. coeliaca. The cell specimens were prepared in accordance with the method previously described (Biken J. 18, 77-92, 1975, Infect. Immun. 38, 817-824, 1982), that is, bacterial cells grown under appropriate cultural conditions were disrupted with either a Braun mechanical cell homogenizer (model MSK; B. Braun Apparatebau) or a Dyno-Mill (type KDL; Willy A, Biochofen Manufactureing Engineers). A crude cell wall fraction obtained by differential centrifugation of a disrupted cell suspension was purified and prepared by removal of components noninherent in cell walls with protease treatment.

[0034]

Peritoneal macrophages of each mouse were cultured for 24 hours in the presence of various concentrations (0, 0.1, 1, 10 or 100 μg/ml) of said preparations and stimulated, then concentration of tumor necrosis factor (TNF-α) released from each macrophage was measured by ELISA (Fig. 5). By these results, it has been found that production of TNFα in response to cell wall components derived from Gram-positive bacteria was more impaired in macrophages of TLR2 knockout mice than in those of wild-type and TLR4 knockout mice.

[0035]

Example 6 (Responsiveness of TLR2 knockout mice to cell wall components of Gram-positive bacteria)

Next, it was investigated that which cell wall component

of Gram-positive bacteria activated macrophages via TLR2. As it has been reported that both p ptidoglycan, which is a cell wall component of Gram-positive bacteria, and lipoteichoic acid (LTA) activate monocytes / macrophages (Infect. Immun. 60, 3664-3672, 1992, Immunity 1, 509-516, 1994, J. Biol. Chem. 271, 23310-23316, 1996, Infect. Immun. 64, 1906-1912, 1996), production amounts of IL-6 and NO_2^- in response to peritoneal macrophages of various kinds of mouse were measured in accordance with the same method as in example 3, with 10 μg/ml of Staphylococcus aureus PGN (Fluka; Fig. 6A) or 10 μg/ml of Staphylococcus aureus LTA (Sigma; Fig. 6C). Further, production of TNFα in peritoneal macrophages of various kinds of mouse in response to PGN (Fig. 6B) or LTA (Fig. 6D) were measured in accordance with the same method as in example 5.

[0036]

The results shown in Fig. 20A indicates that: peritoneal macrophages of wild-type and TLR4 knockout mice produced IL-6 in response to PGN, in contrast, those of TLR2 knockout mice produced no IL-6; NO_2^- was produced when peritoneal macrophages of wild-type and TLR4 knockout mice were cultured with PGN in the presence of IFNγ, in contrast, no NO_2^- was produced when those of TLR2 knockout mice were used; IL-6 was produced in peritoneal macrophages of wild-type and TLR2 knockout mice in response to LTA, in contrast, no IL-6 was produced in those of TLR4 knockout mice (Fig. 6C); NO_2^- was produced when peritoneal macrophages of wild-type and TLR2 knockout mice were cultured with LTA in the presence of IFNγ, in contrast, no NO_2^- was produced when those of TLR4 knockout mice were used (Fig. 6C).

[0037]

As shown in Fig. 6B, peritoneal macrophages of TLR4

knockout mice, as well as those of wild-type mice, increased production of TNFα in response to PGN in a dose-dependent manner, in contrast, those of TLR2 knockout mice showed substantial impairment in production of TNFα, indicating that TLR2 knockout mice were unresponsive to PGN. As shown in Fig. 6D, peritoneal macrophages of TLR2 knockout mice, as well as those of wild-type mice, induced production of TNFα in response to LTA in a dose-dependent manner, in contrast, no TNFα was produced in those of TLR4 knockout mice, indicating that TLR4 knockout mice were unresponsive to LTA. These results demonstrate that PGN, which is a cell wall component of Gram-positive bacteria, activates macrophages via TLR2, and that LTA activates macrophages via TLR4.

[0038]

Example 7 (In vitro kinase assay and Western blot analysis)

TLR family members are known as intracellular signaling molecules which activate a serine-threonine kisase IRAK via an adapter protein MyD88, and subsequently activate rel-type transcription factor, NF-$_\kappa$B (Mol. Cell 2, 253-258, 1998, J. Exp. Med. 187, 2097-2101, 1998, Immunity 11, 115-122, 1999). Whether LPS and PGN activate the intracellular signaling molecules was examined as follows: peritoneal macrophages (1×10^6) of various kinds of mouse were stimulated with 1 ng/ml of LPS of Salmonella minnesota Re-595 or 1 μg/ml of PGN of Staphylococcus aureus for the period indicated in Fig. 7; these bacterial cell components were lysed in lysis buffer (buffer containing Triton X-100 at a final concentration of 1.0 %, 137 mM of NaCl, 20 mM of Tris-HCl, 5 mM of EDTA, 10 % of glycerol, 1 mM of PMSF, 20 μg/ml of Aprotinin, 20 μg/ml of Leupeptin, 1 mM of Na_3VO_4, and 10 mM of β-glycerophosphate; pH 8.0); the cells

w re immunoprecipitated with anti-IRAK antibody (Hayashibara Biochemical Laboratories, Inc.); in vitro kinase assay were conducted as previously described (Biochem. Biophys. Res. Commun. 234, 183-196, 1998, Immunity 11, 115-122, 1999); autophosphorylation of IRAK were measured (Auto shown in Fig. 7A and B).

[0039]

The lysates were dissolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blotted with anti-IRAK antibody (Transduction Laboratories) and visualized by using the enhanced chemiluminescence system (DuPont) (WB in Fig.7A and B). These results show that IRAK activation in response to LPS was observed in wild-type (wild-type) and TLR2 knockout (TLR2-/-) mice, but not observed in TLR4 knockout (TLR4-/-) mice. In contrast, IRAK activation in response to PGN was observed only in wild-type and TLR4 knockout mice. Thus indicates that LPS is recognized via TLR4, and that PGN is recognized via TLR2 respectively.

[0040]

NF-$_\kappa$B activation in response to LPS or PGN was also investigated. Macrophages of various kinds of mouse were stimulated with said LPS or PGN, then nuclear extracts from the macrophages were purified and incubated with a probe specific to DNA binding site of NF-$_\kappa$B, and visualized by electrophoretic mobility shift assay as described previously (Immunity 9, 143-150, 1998). The results are shown in Fig. 7C and D. Arrows in Fig. 21C and D indicate the position of a complex comprised of NF-$_\kappa$B and the specific probe, and arrowheads indicate the position of specific probe only. As a result, DNA

binding activity of NF-$_\kappa$B in response to LPS was detected in nuclear extracts from macrophag s of wild-type and TLR2 knockout mice, but not in those of TLR4 knockout mice. In contrast, NF-$_\kappa$B activation in response to PGN was observed in macrophages of wild-type and TLR4 knockout mice but not in those of TLR2 knockout mice. Thus indicates that TLR4 is essential for LPS-induced NF-$_\kappa$B activation, and that TLR2 is essential for PGN-induced NF-$_\kappa$B activation.

[0041]

[Effect of the Invention]

The TLR2 knockout mouse of the present invention is unresponsive to peptidoglycan which is a cell wall component of Gram-positive bacteria and the like, and hyporesponsive to cell wall fractions of Gram-positive bacteria. Therefore, by using the TLR2 knockout mice of the present invention, it becomes possible to obtain useful information of signaling receptors of selective components such as peptidoglycan which is a cell wall component of Gram-positive bacteria.

[Brief Explanation of Drawings]

[Fig. 1] This is a graph showing gene maps of the TLR2 knockout mice and the wild-type mice of the present invention.

[Fig. 2] This is a graph showing survival indices of the TLR2 knockout mice and the wild-type mice of the present invention having an injection of LPS derived from Escherichia coli.

[Fig. 3] This is a graph showing lipid A- or LPS-induced production amount of IL-6, TNFα or NO_2^- in the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention.

[Fig. 4] This is a graph showing the results of responsiveness of splenic B cells of the TLR2 knockout mice, the wild-type mice

and the TLR4 knockout mice of the pr sent invention to LPS derived from Salmonella minnesota Re-595.

[Fig. 5] This is a graph showing the results of responsiveness of peritoneal macrophages of the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to cell wall fractions of Gram-positive bacteria.

[Fig. 6] This is a graph showing PGN- or LTA-induced production amount of IL-6, NO_2^- or TNFα in the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention.

[Fig. 7] This is a graph showing the results of in vitro kinase assay, Western blot analysis and electrophoretic mobility shift assay in the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention.

[Name of Document] Abstract

[Abstract]

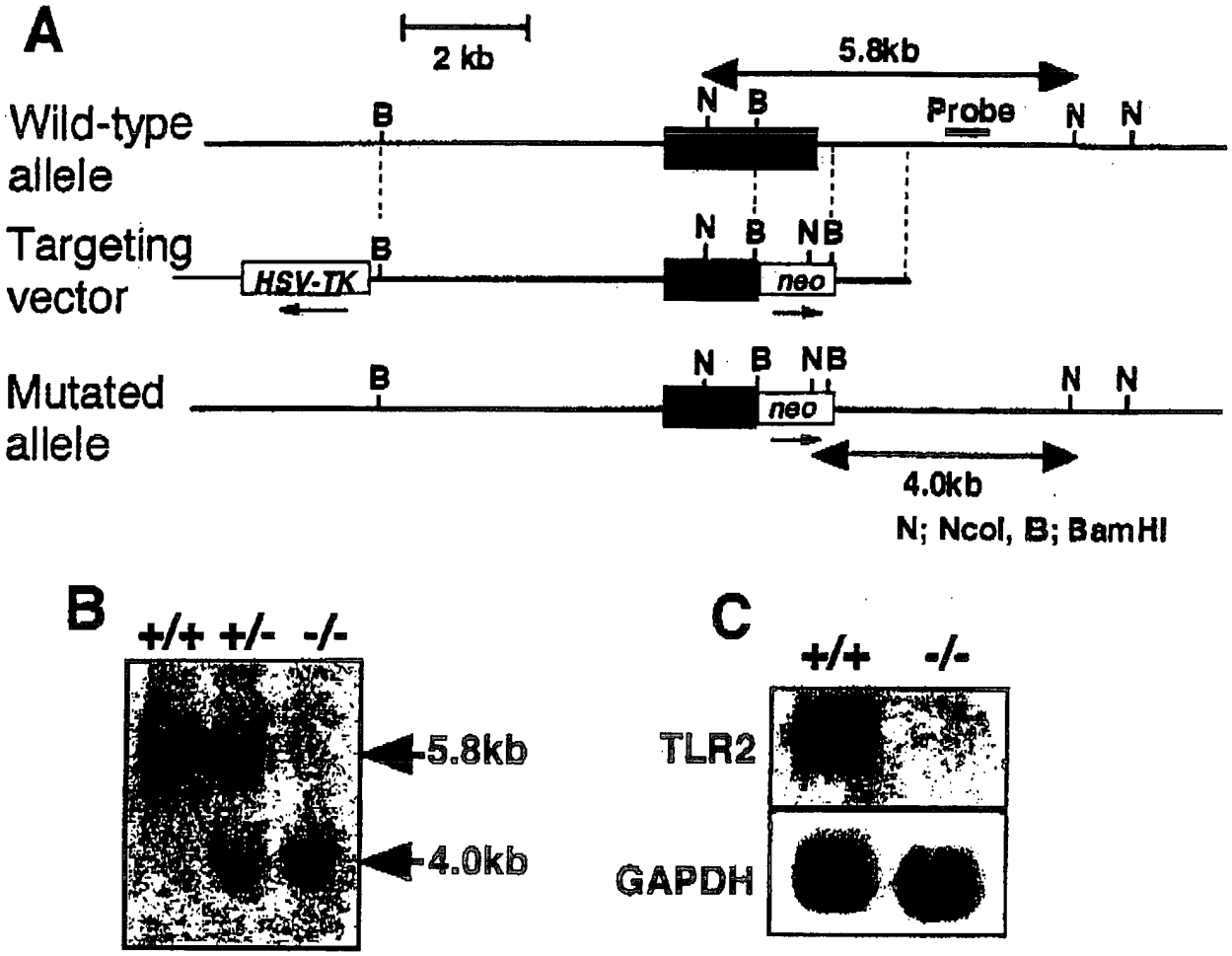
[The Object] The object of the present invention is to provide a knockout mouse which is unresponsive to peptidoglycan, and is useful for elucidating the contribution of individual members of the TLR family to a signaling stimulated with bacterial cell components in vivo, in particular, the role of TLR2 in vivo.

[Solving Means] Generating knockout mice being unresponsive to peptidoglycan by a process comprising the steps of: a targeting vector is constructed by replacing a whole or a part of a gene fragment of an exon region containing a cytoplasmic region of TLR2 gene and the like with a plasmid having a poly A signal and a marker gene; the targeting vector is introduced into an embryonic stem cell; the targeting embryonic stem cell being deficient in function of TLR2 gene is microinjected into the blastocyst of a mouse and the blastocyst is put back into the uterus of a recipient mouse.

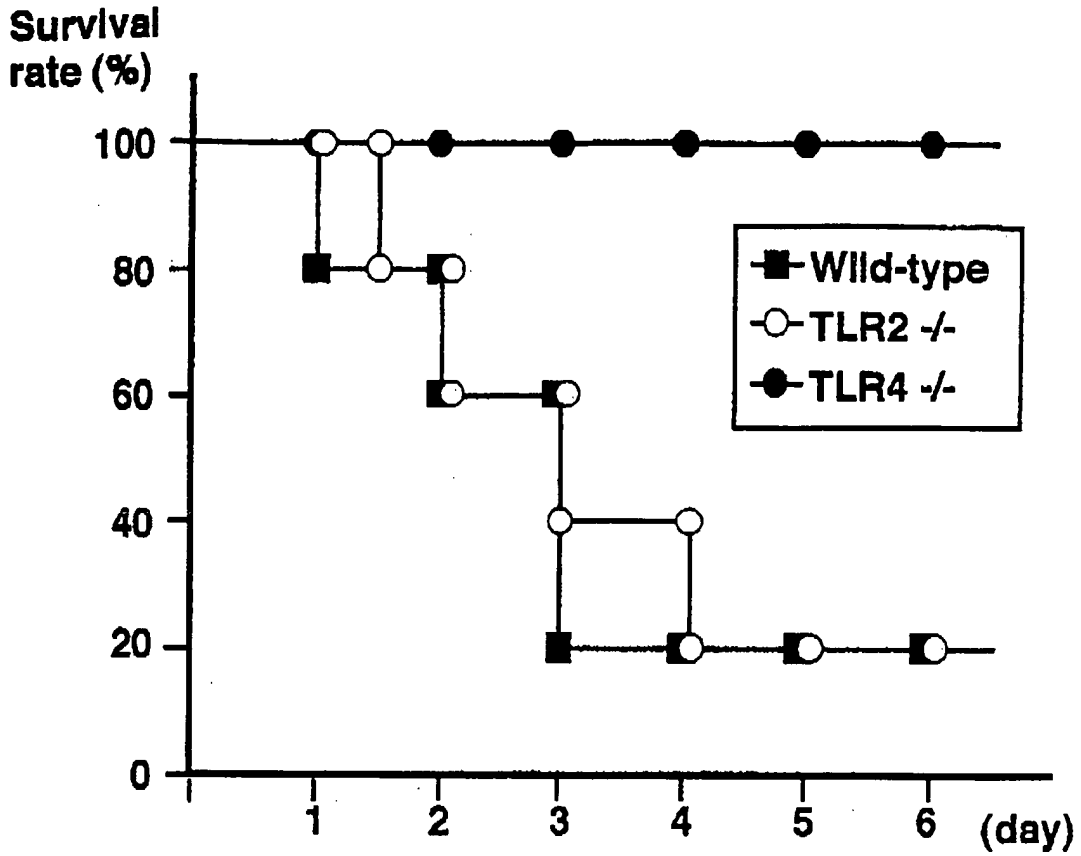
[Selected Drawing] Fig. 1

[Name f Docum nt] Drawing

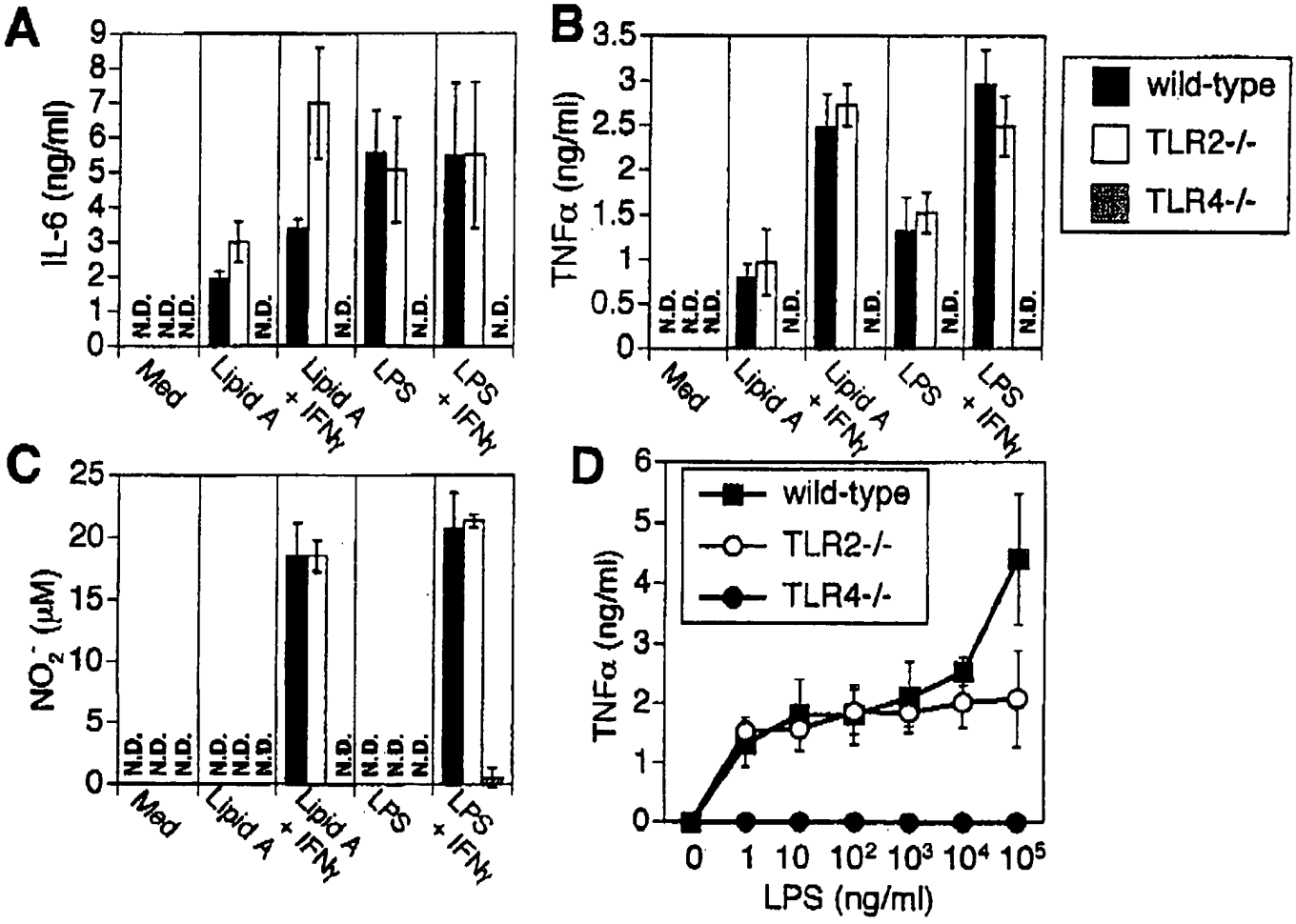
[Fig. 1]



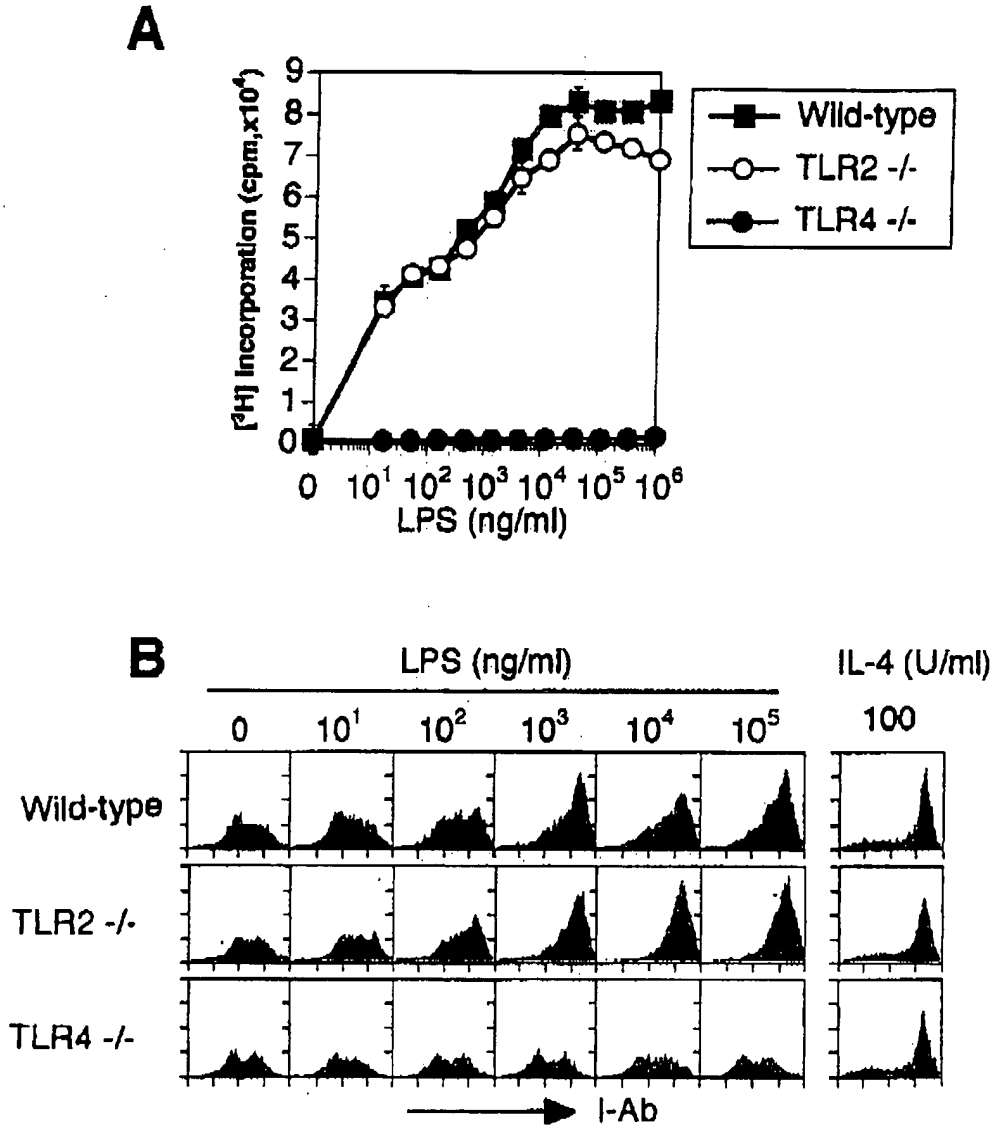
[Fig. 2]


Survival rate (%)
100
80
60
40
20
0
1
2
3
4
5
6
(day)
Wild-type
TLR2 -/-
TLR4 -/-

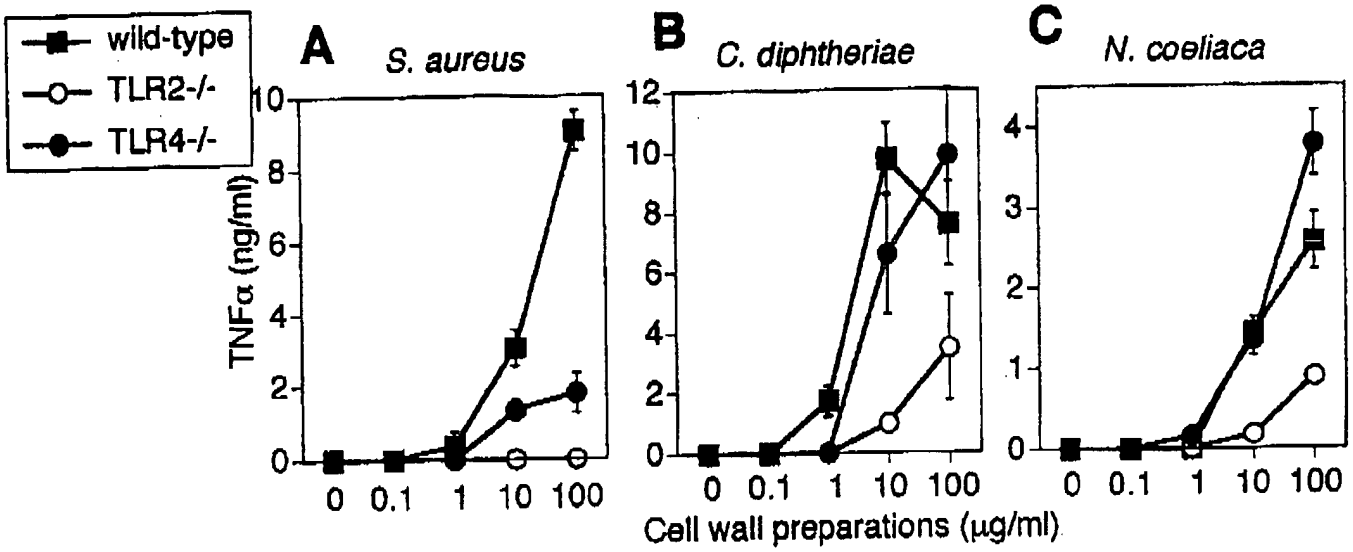
[Fig. 3]


A
IL-6 (ng/ml)
N.D.
Med
Lipid A
Lipid A + IFNγ
LPS
LPS + IFNγ
B
TNFα (ng/ml)
wild-type
TLR2-/-
TLR4-/-
C
NO_2^- (μM)
D
TNFα (ng/ml)
LPS (ng/ml)
0
1
10
10^2
10^3
10^4
10^5

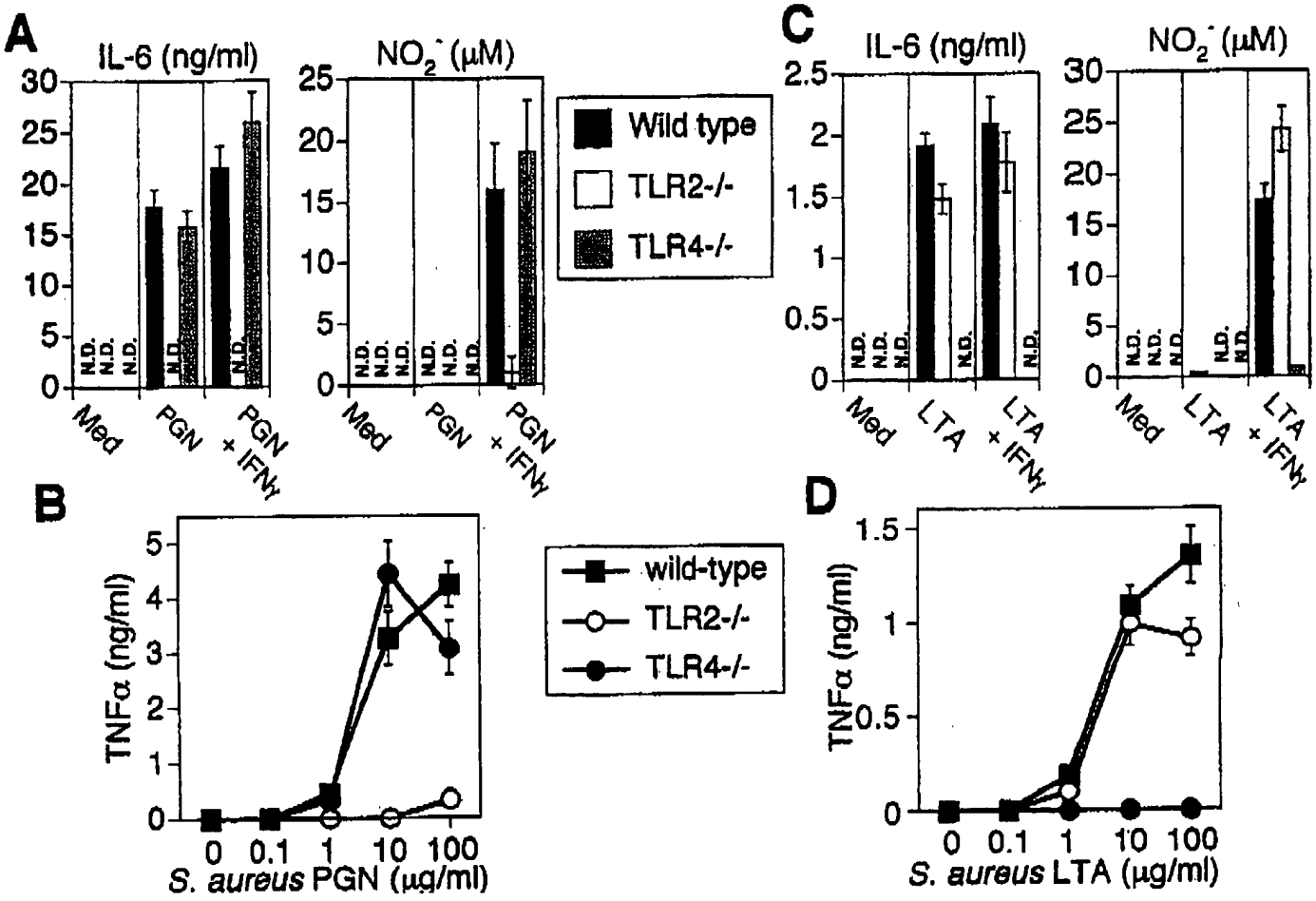
【Fig. 4】


A
[3H] Incorporation (cpm,x10⁴)
9
8
7
6
5
4
3
2
1
0
0 10¹ 10² 10³ 10⁴ 10⁵ 10⁶
LPS (ng/ml)
Wild-type
TLR2 -/-
TLR4 -/-
B
LPS (ng/ml)
IL-4 (U/ml)
0 10¹ 10² 10³ 10⁴ 10⁵
100
Wild-type
TLR2 -/-
TLR4 -/-
I-Ab

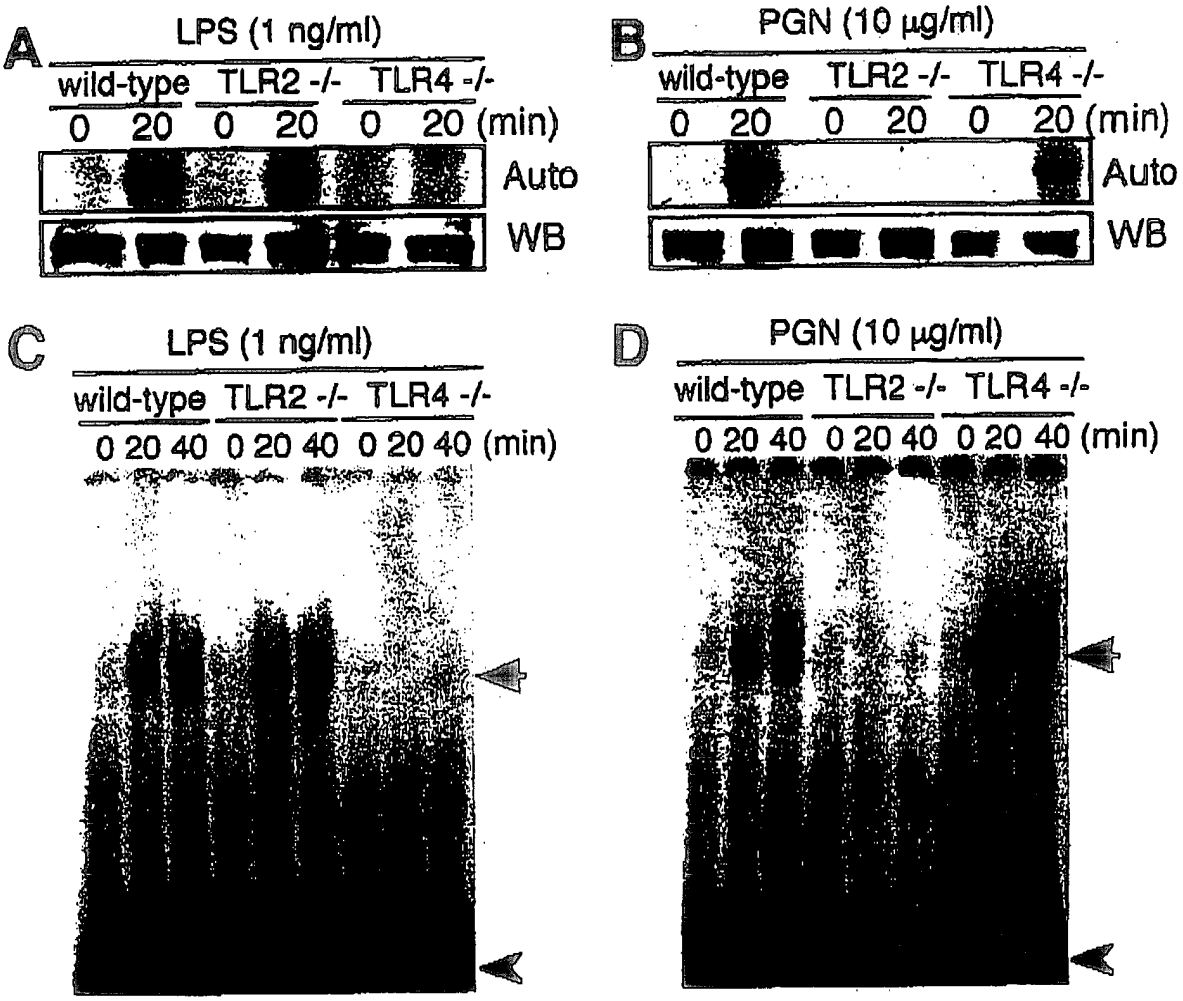
【Fig. 5】


wild-type
TLR2-/-
TLR4-/-
A
S. aureus
B
C. diphtheriae
C
N. coeliaca
TNFα (ng/ml)
Cell wall preparations (μg/ml)

【Fig. 6】


A
IL-6 (ng/ml)
NO2- (μM)
Med
PGN
PGN + IFNγ
Wild type
TLR2-/-
TLR4-/-
C
IL-6 (ng/ml)
NO2- (μM)
Med
LTA
LTA + IFNγ
B
TNFα (ng/ml)
S. aureus PGN (μg/ml)
wild-type
TLR2-/-
TLR4-/-
D
TNFα (ng/ml)
S. aureus LTA (μg/ml)

[Fig. 7]


A
LPS (1 ng/ml)
wild-type
TLR2 -/-
TLR4 -/-
0 20 0 20 0 20 (min)
Auto
WB
B
PGN (10 μg/ml)
wild-type
TLR2 -/-
TLR4 -/-
0 20 0 20 0 20 (min)
Auto
WB
C
LPS (1 ng/ml)
wild-type
TLR2 -/-
TLR4 -/-
0 20 40 0 20 40 0 20 40 (min)
D
PGN (10 μg/ml)
wild-type
TLR2 -/-
TLR4 -/-
0 20 40 0 20 40 0 20 40 (min)